

Soluble Receptor Potentiates Receptor-Independent Infection by Murine Coronavirus

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Mouse hepatitis virus (MHV) infection spreads from MHV-infected DBT cells, which express the MHV receptor CEACAM1 (MHVR), to BHK cells, which are devoid of the receptor, by intercellular membrane fusion (MHVR-independent fusion). This mode of infection is a property of wild-type (wt) JHMV cl-2 virus but is not seen in cultures infected with the mutant virus JHMV srr7. In this study, we show that soluble MHVR (soMHVR) potentiates MHVR-independent fusion in JHMV srr7-infected cultures. Thus, in the presence of soMHVR, JHMV srr7-infected DBT cells overlaid onto BHK cells induce BHK cell syncytia and the spread of JHMV srr7 infection. This does not occur in the absence of soMHVR. soMHVR also enhanced wt virus MHVR-independent fusion. These effects were dependent on the concentration of soMHVR in the culture and were specifically blocked by the anti-MHVR monoclonal antibody CC1. Together with these observations, direct binding of soMHVR to the virus spike (S) glycoprotein as revealed by immunoprecipitation demonstrated that the effect is mediated by the binding of soMHVR to the S protein. Furthermore, fusion of BHK cells expressing the JHMV srr7 S protein was also induced by soMHVR. These results indicated that the binding of soMHVR to the S protein expressed on the DBT cell surface potentiates the fusion of MHV-infected DBT cells with nonpermissive BHK cells. We conclude that the binding of soMHVR to the S protein converts the S protein to a fusion-active form competent to mediate cell-cell fusion, in a fashion similar to the fusion of virus and cell membranes.

The initial step of viral infection is the binding of the virus to its receptor on the target cell. In enveloped viruses, the spike or surface glycoprotein(s), which comprises the virus peplomers, is responsible for this binding. Following binding, the spike glycoprotein(s) mediates the fusion of the viral envelope and cell membrane. At least two different sites for the fusion of viral and cellular membranes have been recognized. In the case of influenza virus, the virion is first incorporated into the endosome by receptor-mediated endocytosis, and subsequently the viral hemagglutinin (HA) is activated by the low-pH environment of the endosome and converted from a nonfusogenic to a fusogenic form. This functional change is accompanied by a conformational change of the HA protein (53). In the case of human immunodeficiency virus (HIV), the virion is thought to enter the cell directly from the cytoplasmic surface membrane via a nonendosomal pathway. Again, HIV envelope protein is converted from a nonfusogenic to a fusogenic form by the binding of its receptor and coreceptor. This is also associated with conformational changes of the envelope protein (43). Through the fusion of the viral envelope and cell membrane, the genetic material of the virus is released into the cell interior and replication is initiated.

The entry pathway of the murine coronavirus mouse hepatitis virus (MHV) has not been well defined. Studies using lysosomotropic agents have suggested either an endosomal or a nonendosomal pathway (29, 35, 37). Recently, Nash and Buchmeier (38) reported that a mutant derived from MHV strain JHMV with low-pH-dependent fusion activity entered

by an endosomal pathway, while the parental JHMV utilized either an endosomal or a nonendosomal pathway, depending on the nature of the cells.

MHV is an enveloped virus with a positive-stranded, nonsegmented genomic RNA of about 32 kb (33). MHV infects cells via MHV-specific receptor proteins. Several different molecules function as MHV receptors (4, 6, 39), among which CEACAM1 (MHVR) is the most prevalent (40, 41). MHVR is an immunoglobulin superfamily protein with 4 or 2 ectodomains. The N-terminal ectodomain of MHVR contains the virus-binding site (15, 16). As has been shown with chimeric MHVR and mouse poliovirus receptor homolog proteins (12) or chimeras of MHVR and human immunoglobulin G (IgG) constant regions (20), the N-terminal ectodomain of MHVR is sufficient for receptor function.

The viral protein that interacts with MHVR is the spike (S) protein. The S protein is synthesized as a 180- to 200-kDa protein that is cleaved into two subunits by host-derived protease (44). The N-terminal subunit, called S1, forms the outermost knob-like structure of the spike, and the C-terminal S2 subunit forms the stem-like structure beneath the knob (11). Each peplomer is supposedly composed of two molecules of the S1–S2 heterodimer. Among other functions (47), the S protein is responsible for receptor binding, and this is mediated by the N-terminal 330 amino acids of the S1 subunit (S1N330) (31, 45). At present, no additional regions are thought to be necessary for the receptor-binding activity. Various regions of the S protein are reported to be critical for entry of the virus into cells (19, 22, 34, 50).

Recently, we have reported that soluble receptor-resistant (srr) mutants derived from wild-type (wt) JHMV bound to a second form of the MHVR, called CEACAM1b (MHVR2), as

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efficiently as did wt virus (36). However, these mutants, in contrast to wt virus, failed to enter cells expressing MHVR2 (36). MHVR2 is derived from MHV-resistant SJL mice, while CEACAM1a (MHVR1) is derived from MHV-susceptible BALB/c mice (14, 55). We assumed that MHVR1, but not MHVR2, is able to activate the JHMV *srr* S proteins and facilitate viral entry into cells (36).

In order to analyze the activation of S protein by MHVR1, we have expressed and purified a soluble form of MHVR (soMHVR) by use of a recombinant baculovirus. soMHVR is considered to interact with S protein similarly to membrane-anchored MHVR1 (40, 42, 56). Furthermore, soMHVR facilitates the study of MHV virus-receptor interaction in cell-free systems. The experimental system we have developed is based on the observation that MHV infects DBT cells expressing MHVR but fails to infect cells that do not express MHVR. However, the infection spreads from MHV-infected DBT cells to MHVR-deficient cells by intercellular fusion (MHVR-independent fusion or infection), as originally reported by Gallagher et al. (21). wt JHMV displays strong MHVR-independent infection, while JHMV *srr7* lacks this mode of infection (49).

In the present study, we show that soMHVR can activate MHVR-independent infection in JHMV *srr7*-infected cultures. This indicates that soMHVR converts a fusion-negative JHMV *srr7* S protein to a fusion-active form. This activation phenotype is not specific to this particular mutant but is also observed in the wt JHMV S protein. Detailed examination of this system should provide further insights into the conformational changes associated with the fusogenic activation of the MHV S protein.

MATERIALS AND METHODS

Cells and viruses. DBT cells expressing MHVR1 (32), as well as BHK 13 (BHK) cells devoid of MHVR, were grown in Dulbecco's minimal essential medium (DMEM; Nissui, Tokyo, Japan) supplemented with 5% fetal bovine serum (FBS; Gibco BRL, Grand Island, N.Y.) (DMEM-FBS). DBT cells were used for MHV infection, titration, and propagation. BHK cells nonpermissive to MHV were target cells for MHVR-independent infection as described previously (49). The wt MHV strain JHMV cl-2 (51) and its mutant JHMV *srr7* (42) were propagated in DBT cells, and the supernatants of culture fluids were used for infection as previously reported (52). *srr7* has a mutated amino acid at position 1114 (Leu to Phe) in the S protein compared with wt virus. A recombinant vaccinia virus, vTF7.3, harboring the T7 RNA polymerase gene was provided by B. Moss (18) and was used to express the MHV S protein as described previously (42).

Assay of virus infectivity. To examine virus infectivity, we have utilized a new assay method. DBT cells cultured in 24-well plates (Iwaki, Tokyo, Japan) were inoculated with virus and cultured in DMEM-FBS containing 0.5% methylcellulose (Sigma, St Louis, Mo.) at 37°C for 12 to 24 h. The cells were stained with crystal violet after fixation with formalin. The syncytia counted under a microscope (CK30; Olympus, Tokyo, Japan) were shown as PFU. This assay was slightly more sensitive for virus titration than the plaque assay using 6-well plates, in which cells were stained with neutral red (26, 52).

To measure virus titers, cells as well as culture fluids were collected in 1.5-ml tubes and sonified (Olympus) for 3 min at level 3. This ruptured the cultured cells but did not influence MHV infectivity. After spinning at 10,000 rpm at 4°C for 5 min, the virus titers in the supernatants were determined as described above.

Viral neutralization assay. To examine the virus neutralization (VN) titer of soMHVR, approximately 200 PFU of wt JHMV cl-2 in 25 μ l was mixed with an equal volume of soMHVR serially diluted with DMEM-FBS and the mixture was incubated at room temperature (RT, 22 $\pm$ 2°C) for 50 min. Twenty microliters of the mixture was inoculated onto DBT cells prepared in 24-well plates, and nonneutralized virus titers were determined as described above. The highest dilution of soMHVR to neutralize more than 50% of virus infectivity, compared

to virus incubated in the absence of soMHVR, was considered to contain 1 VN unit. soMHVR was produced by a recombinant baculovirus and purified as described below.

To examine the resistance of wt JHMV cl-2 and JHMV *srr7* to neutralization by soMHVR, we have carried out a viral neutralization test essentially as described previously (42). Approximately 10⁵ PFU of virus in 25 μ l of DMEM-FBS was mixed with an equal volume of soMHVR (5,000 VN units) and incubated at 37°C or RT for various lengths of time. Residual infectivity was then measured by the assay as described above.

Recombinant baculovirus for soMHVR expression. soMHVR was expressed using a baculovirus expression system. The pT7-soMHVR1-HA expression vector encoding soluble CEACAM1a (4), which consists of the first and fourth ectodomains (40), was used as a template DNA to produce an soMHVR construct with three different tags at the C terminus, i.e., an influenza HA epitope, myc, and six repeats of histidine. Thus, pT7-soMHVR1-HA was used as a PCR template with two primers, MHVR-5' (40) and HA-myc-His (5' TCAATGGT GATGGTGATGGTGAGATCCTCTCTCTGAGATGAGTTTGTTCAGC ATAATCTGGAAC-3'), and Ex-taq polymerase (Takara, Tokyo, Japan) as described previously (40). The amplified DNA fragment was inserted into the pTarget T vector (Promega, Madison, Wis.) under the control of the T7 promoter. From this plasmid, an *Eco*RI-to-*Sma*I fragment, which contained the entire soMHVR gene together with the three tags, was isolated and inserted into the *Eco*RI-*Sma*I site of the baculovirus transfer vector pVL1392 (kindly provided by Y. Matsuura). A recombinant baculovirus able to express soMHVR was recovered from Sf9 cells with a kit (BaculoGold DNA; PharMingen, San Diego, Calif.) according to the manufacturer's instructions. The recombinant baculovirus was shown by indirect immunofluorescence using an anti-HA antibody (mouse monoclonal antibody [MAb] clone 12CA5; Boehringer, Mannheim, Germany) and anti-mouse IgG labeled with fluorescein isothiocyanate (Cappel Organon Teknika, Durham, N.C.) to express soMHVR in virus-infected cells. The selected virus was plaque purified in Sf9 cells three times and used to express soMHVR.

Expression and purification of soMHVR. soMHVR was expressed in Tn5 cells by the recombinant baculovirus (Bac-soR1-HmH) prepared as described above. Tn5 cells were infected with Bac-soR1-HmH at a multiplicity of infection (MOI) of 1 or higher and then incubated at 26°C for 1 h. Cells were cultured with Ex-cell 405 medium (Gibco BRL) at 26°C for 3 days. The culture fluids, normally 100 to 200 ml, were centrifuged at 15,000 rpm for 30 min (CR 26H centrifuge; Hitachi, Tokyo, Japan) and clarified culture fluids were mixed with polyethylene glycol 6000 at a final concentration of 30%. Following incubation at 4°C for 1 to 2 h, the mixture was centrifuged at 10,000 rpm for 30 min, and the resultant precipitate was dissolved in a small volume (10 to 20 ml) of a lysis buffer (50 mM NaH₂PO₄, 300 mM NaCl, 10 mM imidazole [pH 8.0]). soMHVR with a six-His tag was purified by Ni-nitrilotriacetic acid (Qiagen, Hilden, Germany) affinity chromatography according to the manufacturer's instructions. The concentration of soMHVR was determined by enzyme-linked immunosorbent assay using the HA peptide (Boehringer) as a control. The purity of the expressed soMHVR was examined by Western blot analysis as described previously (46).

MHVR-independent infection. MHVR-independent infection of BHK cells was carried out essentially as described previously (49). Confluent DBT cells in 35-mm dishes (Iwaki) were infected with MHV at an MOI of 1. After a 1-h incubation at 37°C, cells were incubated with DMEM-FBS at 37°C for 3 to 4 h. Then these cells were treated with trypsin to produce a single-cell suspension in DMEM-FBS. Ten microliters of the suspension containing 10⁴ DBT cells was overlaid onto confluent BHK cells (8 $\times$ 10⁵ to 10 $\times$ 10⁵) cultured in 200 μ l of DMEM-FBS in collagen-treated 24-well plates (Iwaki). Mixed cells were incubated at 37°C for a further 12 to 24 h in the presence or absence of soMHVR. At this time, fused BHK cells were observed under the microscope. The mixed cell culture was then fixed with 5% formalin and stained with 0.1% crystal violet dissolved in 50 mM boric acid. Syncytia were counted as plaques of MHVR-independent infection.

In some experiments, various amounts (0 to 6 μ g/ml) of an MHVR-specific MAb, CC1 (kindly provided by K. Holmes) (15, 54), were mixed with soMHVR1 prior to overlaying the cells. Two hundred microliters of 80 nM soMHVR was mixed with various amounts of CC1 and incubated at RT for 1 h. BHK cells in 24-well plates were then overlaid with the preincubated mixture together with 10⁴ *srr7*-infected DBT cells. After 12 to 15 h of culture, the number of syncytia was counted. As a control, soMHVR was mixed with DMEM-FBS containing no CC1.

Coimmunoprecipitation. Coimmunoprecipitation was performed to allow observation of the direct binding of soMHVR and S protein expressed on DBT cells. A total of 3 $\times$ 10⁶ DBT cells prepared in a 35-mm dish were infected with JHMV *srr7* at an MOI of 1 and cultured at 37°C for 12 h. These cells were

incubated at 37°C for 15 min in the presence of various concentrations of soMHVR. After four washes with phosphate-buffered saline, pH 7.2 (PBS), cells were collected with a rubber policeman, lysed with a buffer (PBS containing 0.65% Nonidet P-40), and spun at 15,000 rpm at 4°C for 10 min. Supernatants were incubated for 1 h at RT with anti-soMHVR (the anti-HA MAb 12CA5) which had been coupled to protein A-Sepharose CL-4B (Pharmacia, Uppsala, Sweden). After a wash with PBS, each sample was treated with electrophoresis sample buffer and electrophoresed on a sodium dodecyl sulfate (SDS)-polyacrylamide gel as described elsewhere (46). Coimmunoprecipitated S proteins were analyzed by Western blotting using the anti-JHMV S MAb 30B, a gift from Stuart Siddell. JHMV S proteins were detected by enhanced chemiluminescence (ECL; Amersham, Arlington Heights, Ill.) as described previously (42).

Expression of MHV S proteins in BHK cells. MHV S proteins were expressed in BHK cells by a transient vaccinia virus expression system as previously reported (18, 36, 42). BHK cells cultured in 60-mm dishes (Iwaki) were infected with vTF7.3, a recombinant vaccinia virus harboring the T7 RNA polymerase gene (18). After 1 h at 37°C, the cells were trypsinized and transfected with plasmid pTarget cl-2S or pTarget srr7-S, containing, respectively, the wt JHMV S protein gene (48) or the JHMV srr7 S protein gene under the control of the T7 promoter (36), by electroporation using a Gene Pulser (Bio-Rad, Hercules, Calif.) (42). Cells were cultured in the presence or absence of soMHVR in DMEM-FBS at 37°C for various lengths of time in order to observe syncytium formation.

Fusion assay. The fusion activities of S proteins were quantified by determination of luciferase activity expressed from the pT7EMCLuc plasmid (pTM3-luc), which harbors the firefly luciferase gene under the control of the T7 promoter and 5' untranslated region (5' UTR) of encephalomyocarditis virus (2). One group of BHK cells that had been infected with vTF7.3 at an MOI of 3 to 5 was transfected with either the pTarget cl-S, pTarget srr7-S, or pTarget vector by electroporation (42). A total of 5×10^5 cells were distributed in a collagen-treated 24-well plate (Iwaki) and incubated at 37°C for 7 to 9 h in the presence or absence of 150 nM soMHVR. A second group of BHK cells that had been transfected with plasmid pTM3-luc (2), kindly provided by Y. Matsuura, by use of Lipofectamine (Gibco BRL) was infected with wt vaccinia virus strain WR at an MOI of 3 to 5 and cultured for 9 to 11 h. These cells, 5×10^5 in number, were overlaid onto the first group of BHK cells prepared in 24-well plates. They were incubated at 37°C for 4 h. If the S protein expressed in a well permits cell-cell fusion, then luciferase is expressed from the pTM3-luc plasmid. Luciferase activities in each well were measured with the PiCa gene kit (Toyoh Ink, Tokyo, Japan) according to the procedure recommended by the manufacturer by use of a luminometer (Microtech Niton, Tokyo, Japan).

RESULTS

Potential of MHVR-independent fusion by soMHVR.

The soMHVR used in these studies was shown by Western blot analysis to have an apparent molecular size of approximately 44 kDa (Fig. 1). The purified material was essentially homogeneous, although staining with the anti-myc antibody revealed some evidence of degradation. The final concentration of the concentrated and purified soMHVR1 was 3 μ M, and it contained 5,000 VN units. Its specific VN activity (VN units per protein concentration) was roughly equivalent to that of the soMHVR reported by Zelus et al. (56).

DBT cells infected with wt JHMV cl-2 or its mutant JHMV srr7 at an MOI of 1 were incubated for 4 h and treated with trypsin, and 10^4 cells in 10 μ l of DMEM were mixed into 200 μ l of culture medium overlaying a BHK cell monolayer prepared in 24-well plates. As shown in Fig. 2, JHMV cl-2-infected DBT cells induced fusion of BHK cells, while no syncytia were visible as long as 48 h after overlay when JHMV srr7-infected DBT cells were overlaid onto BHK cells (Fig. 2A and C), thus confirming our previous observation (49). If we added 10 μ l of soMHVR, which resulted in 150 nM soMHVR in the culture fluid, a large number of syncytia were observed in BHK cells overlaid with JHMV srr7-infected DBT cells. This induction of syncytia by soMHVR occurred when soMHVR was added to

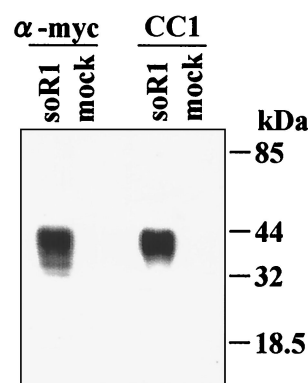


FIG. 1. Western blot analysis of purified soMHVR. soMHVR expressed and purified as described in Materials and Methods was electrophoresed in an SDS-10% polyacrylamide gel and transferred onto a nitrocellulose membrane. soMHVR was detected by ECL with an anti-myc MAb (α -myc) or MAb CC1, specific to MHVR, and anti-mouse IgG labeled with horseradish peroxidase. As a control, medium from Tn5 cells mock infected with baculovirus (mock) was concentrated and purified similarly to medium from recombinant baculovirus-infected cells (soR1).

DBT cells by 12 h after srr7 infection, but not later than 16 h. In contrast, there was no substantial increase in the number of syncytia when JHMV cl-2-infected DBT cells were overlaid (Fig. 2B and D and Fig. 3). However, the relative syncytium size produced in JHMV cl-2 infection in an MHVR-independent fashion was 2.47 ± 0.59 in the presence of soMHVR, which is significantly larger ($P < 0.001$ by the Student *t* test) than the syncytium size (1.0 ± 0.24) produced in the absence of soMHVR (Fig. 2A and B). Mock-infected DBT cells overlaid onto BHK cells failed to produce syncytia in the presence or absence of soMHVR. Furthermore, virus titers in the BHK cells overlaid with JHMV srr7-infected DBT cells were 1 to 1.5 log₁₀ units higher when cells were cultured in the presence of soMHVR than when they were cultured in the absence of soMHVR (data not shown). This indicated that soMHVR enhanced the MHVR-independent spread of srr7. These results suggested that soMHVR enhanced or potentiated the MHVR-independent fusion activity and infection of the wt JHMV cl-2 and mutant JHMV srr7 S proteins.

soMHVR concentration-dependent effect on MHVR-independent fusion. We examined the effect of soMHVR concentration on MHVR-independent fusion using JHMV srr7. BHK monolayers were cultured in 24-well plates with 200 μ l of medium together with 10^4 srr7-infected DBT cells as described above. Ten microliters of soMHVR at various concentrations was added, and cultures were incubated at 37°C for 12 h. As shown in Fig. 4, the number of syncytia increased in proportion to the increasing soMHVR concentration. It was also observed that syncytia were larger in the presence of higher concentrations of soMHVR (data not shown). At a concentration of about 25 nM soMHVR, the number and size of syncytia reached a plateau. This observation also suggested that soMHVR is responsible for the induction of MHVR-independent fusion.

Effect of the anti-MHVR MAb CC1 on soMHVR-mediated MHVR-independent fusion. To see whether the observed effect is related to direct binding of soMHVR to the S protein on

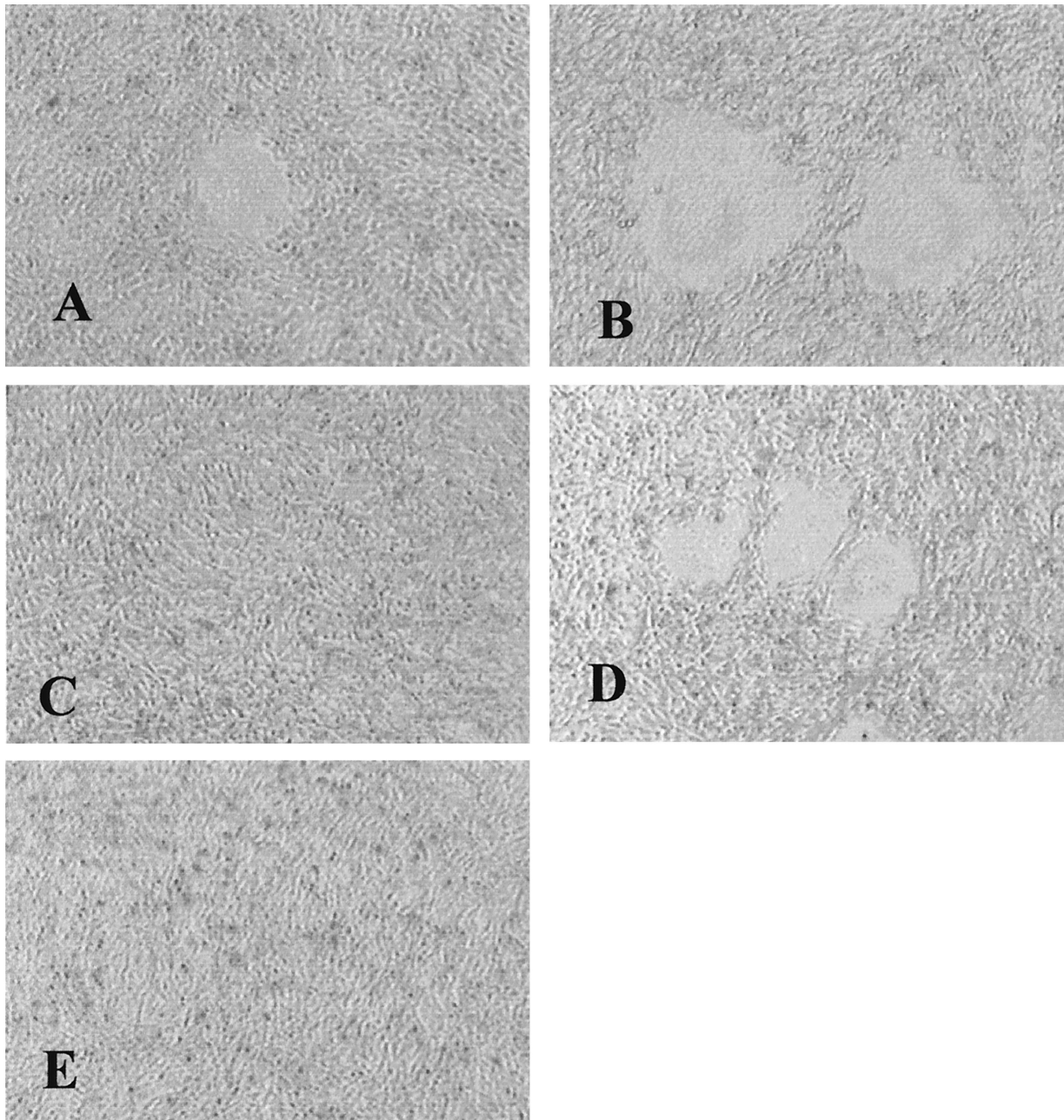


FIG. 2. Induction of *srr7*-mediated MHVR-independent fusion by soMHVR. Confluent BHK cells in a 24-well plate were overlaid with DBT cells infected with wt JHMV cl-2 (A and B) or mutant JHMV *srr7* (C and D) and incubated in the presence (B and D) or absence (A and C) of 150 nM soMHVR. As a control, BHK cells were overlaid with uninfected DBT cells in the presence of soMHVR (E). Cells were observed under a microscope at 12 h after overlay.

the DBT cell surface, we blocked the binding of soMHVR to the S protein with a MAb specific to MHVR, CC1. This MAb recognizes the virus-binding region of the MHVR and hence inhibits the binding of MHVR to the MHV S protein (15, 54). soMHVR, at a concentration of 80 nM, was mixed with 0 to 6 μ g of CC1/ml and incubated at RT for 1 h. This mixture, together with 10^4 JHMV *srr7*-infected DBT cells, was overlaid onto BHK cells in 24-well plates. The number of syncytia was counted 12 h later. As shown in Fig. 5, formation of syncytia was blocked by CC1 in a concentration-dependent manner. CC1 at 6 ng/ml completely blocked the MHVR-independent

fusion caused by 80 nM soMHVR. This clearly demonstrates that MHVR-independent fusion is mediated by soMHVR bound to the S protein.

Since cells expressing soMHVR have been reported to be susceptible to MHV infection (13), it could also be argued that MHVR-independent fusion in the presence of soMHVR is mediated by soMHVR bound to the BHK cell membrane. In other words, soMHVR could work similarly to membrane-anchored MHVR. BHK cells pretreated for 12 h with 150 nM soMHVR were infected with cell-free JHMV (wt and *srr7*) or overlaid with JHMV *srr7*-infected DBT cells, and formation of

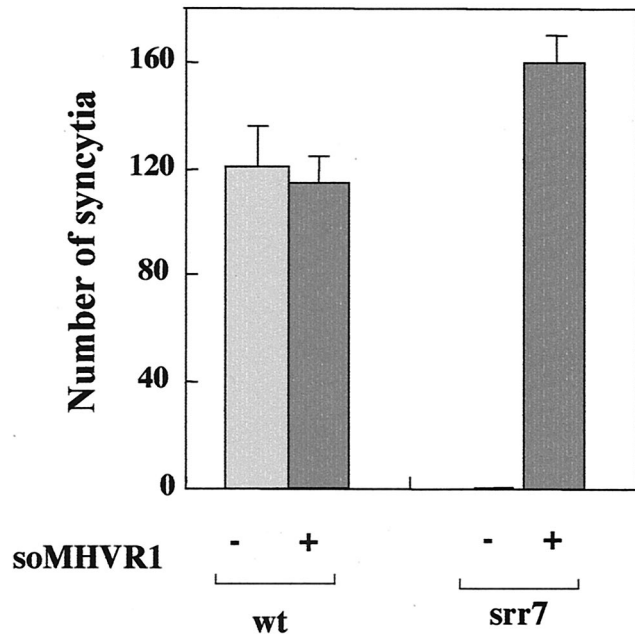


FIG. 3. Effect of soMHVR on fusion formation by MHV-infected DBT cells. BHK cells were overlaid with 10^4 DBT cells infected with wt JHMV or JHMV srr7 and then incubated at 37°C for 12 h in the presence or absence of 150 nM soMHVR. The number of syncytia was counted after staining with crystal violet. Error bars, standard deviations of three independent samples.

syncytia was examined. However, this treatment did not render BHK cells susceptible to MHV infection or MHVR-independent fusion (data not shown). These results exclude the possibility that membrane-associated soMHVR can serve as a receptor for JHMV srr7 infection of BHK cells.

Direct binding of soMHVR to the MHV S protein. In an effort to learn whether soMHVR binds to the S protein expressed on DBT cells, we tried in vain to detect direct binding of soMHVR to the S protein expressed on DBT cells cultured

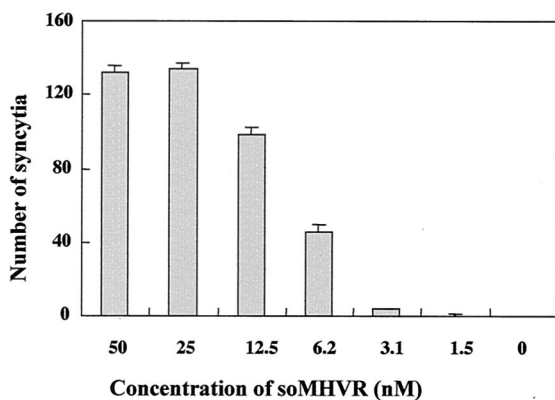


FIG. 4. Effect of soMHVR concentration on MHVR-independent fusion. BHK cells in a 24-well plate were overlaid with 10^4 DBT cells infected with JHMV srr7 and incubated in the presence of various concentrations of soMHVR for 12 h. The number of syncytia was counted after staining with crystal violet. Error bars, standard deviations of three independent samples.

in 24-well plates (1×10^4 to 10×10^4 cells) by coimmunoprecipitation. This was probably due to the fact that the amounts of S protein expressed on DBT cells were below the level needed for detection. Therefore, we scaled up the cell number. We prepared 3×10^6 cells in a 35-mm dish and infected them with srr7 at an MOI of 1. After 12 h of incubation, cells were treated with various amounts of soMHVR for 15 min at 37°C ; soMHVR added at 12 h postinfection (i.e., 8 to 9 h after overlay) induced syncytia. Then soMHVR bound to cells was precipitated from the cell lysate with anti-soMHVR (anti-HA MAbs). Coimmunoprecipitated JHMV srr7 S protein was analyzed by Western blotting as described in Materials and Methods. As shown in Fig. 6, JHMV srr7 S proteins were coimmunoprecipitated with anti-soMHVR. Amounts of precipitated S protein roughly correlated to the amounts of soMHVR added in the culture medium of srr7-infected DBT cells. This result demonstrated that soMHVR directly bound to the S protein, most probably to the S protein expressed on the cell membrane. This finding, together with the inhibition of MHVR-independent fusion by CC1, confirmed that the binding of soMHVR to the S protein is responsible for the induction of MHVR-independent fusion.

Soluble receptor resistance of JHMV srr7. In a previous paper, we showed that JHMV srr7 was resistant to neutralization by soMHVR when incubated at RT for 50 to 60 min (42). In the present study, activation of JHMV srr7 MHVR-independent fusion by soMHVR was detected when cells were incubated at 37°C for as long as 12 h. Thus, we examined whether JHMV srr7 was resistant to neutralization by soMHVR after incubation at 37°C under conditions where soMHVR activated JHMV srr7 fusion activity. Ten microliters of JHMV srr7 and the same volume of soMHVR (5000 VN units; $3 \mu\text{M}$) were mixed and incubated at 37°C or RT for 50 min, and residual infectivity was measured. As a control, wt JHMV cl-2, which is not soluble receptor resistant (42), was similarly treated. As shown in Fig. 7, JHMV srr7 was fully resistant to neutralization by soMHVR at RT, although more than 95% of JHMV srr7 infectivity was neutralized by incubation at 37°C for 1 h. Incubation at 37°C for 2 h reduced srr7 infectivity by more than 3 orders of magnitude. In contrast, wt JHMV cl-2 was neutralized at both 37°C and RT, although neutralization was 3.5 times greater at 37°C than at RT. These data are consistent with our previous results (42) but additionally indicate that JHMV srr7 is not resistant to soMHVR when treated at 37°C .

soMHVR-mediated activation or enhancement of fusion formation by BHK cells expressing S protein. The fact that soMHVR mediates fusion in an MHVR-independent manner predicts that soMHVR would also activate the fusion of BHK cells expressing the MHV S protein. To examine this possibility, we transfected plasmids harboring either wt JHMV or JHMV srr7 S genes by electroporation and expressed these S proteins on BHK cells using a recombinant vaccinia virus, vTF7.3. At intervals after transfection, cells cultured at 37°C in the presence or absence of soMHVR were checked for syncytium formation. Syncytia were observed when the JHMV wt S protein was expressed in BHK cells, even in the absence of soMHVR. This result is consistent with our previous observation that JHMV wt S protein expressed in MHVR-deficient cells induced fusion (48). When cells were cultured in the

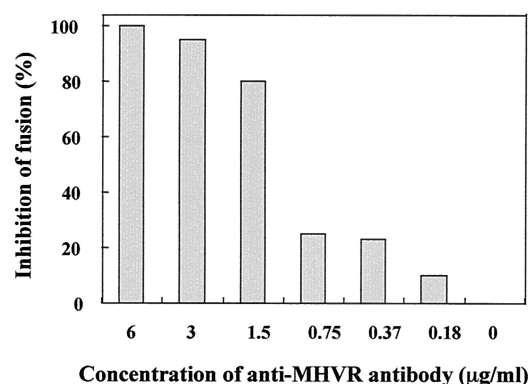


FIG. 5. Effect of the anti-MHVR MAb CC1 on MHVR-independent fusion induced by soMHVR. soMHVR, at an 80 nM concentration in 200 µl of DMEM-FBS, was mixed with different concentrations of MAb CC1 and incubated at RT for 1 h. BHK cells in 24-well plates were then overlaid with this mixture together with 10^4 JHMV srr7-infected DBT cells. After 12 h of cultivation, the number of syncytia was counted. As a control, soMHVR was mixed with DMEM-FBS containing no CC1. Results are representative of multiple independent experiments.

presence of 150 nM soMHVR, fusion formation was apparently enhanced in cells expressing JHMV wt S protein. In cells expressing JHMV srr7 S protein, fusion formation was not observed in the absence of soMHVR, but it was clearly observed in the presence of soMHVR.

To quantify the enhancement of fusion activity by soMHVR, we have used plasmid pTM3-luc, which harbors the luciferase gene as a reporter under the control of the T7 promoter and the encephalomyocarditis virus 5' UTR. BHK cells that had been infected with vTF7.3 and transfected with a plasmid containing either the wt JHMV S gene or the JHMV srr7 S gene under the control of the T7 promoter were mixed with another group of BHK cells transfected with pTM3-luc. They were incubated at 37°C in the presence or absence of 150 µM soMHVR. Luciferase activity expressed as a result of the fusion of two distinct types of BHK cells was measured. As shown in Fig. 8, luciferase activities in cells transfected with the JHMV srr7 S gene were significantly higher ($P < 0.001$ by the Student *t* test) when cells were cultured in the presence of soMHVR than when they were cultured in the absence of soMHVR. Enhancement of fusion activity by soMHVR was also observed in cells transfected with the wt JHMV S gene. Luciferase activities in cells transfected with the wt JHMV S gene and cultured in the presence of soMHVR were approximately threefold higher ($P < 0.001$) than those in cells cultured in the absence of soMHVR. Thus, it was evident that soMHVR induced syncytium formation by BHK cells expressing JHMV srr7 S proteins. Again, in this system, an effect of soMHVR on fusion formation was also observed in cells expressing the wt JHMV S protein. These observations clearly demonstrated that soMHVR activated or enhanced the fusion activity of S protein in BHK cells.

DISCUSSION

Viral receptors are major determinants of cell and tissue-specificity, and the study of virus-receptor interactions is of

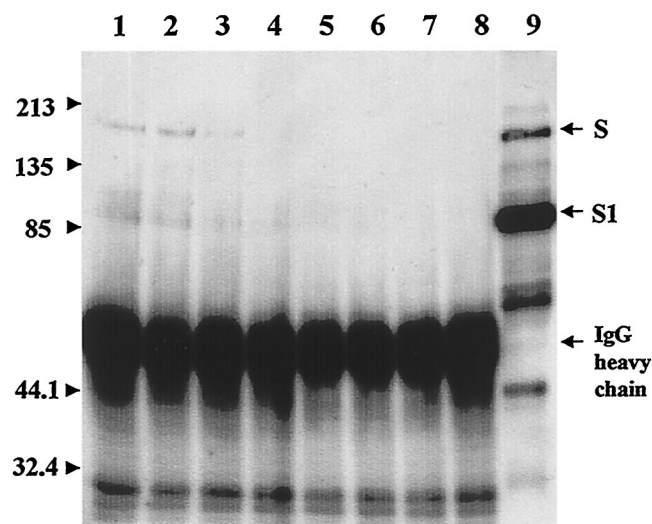


FIG. 6. Direct binding of soMHVR to the JHMV srr7 S protein as examined by coimmunoprecipitation. DBT cells infected with srr7 at an MOI of 1 were incubated with soMHVR at 300 (lane 1), 30 (lane 2), 3 (lane 3), 0.3 (lane 4), 0.03 (lane 5), or 0 (lane 6) nM at 12 h postinfection for 15 min at 37°C. Mock-infected DBT cells were also incubated under the same conditions with 300 (lane 7) or 0 (lane 8) nM soMHVR. Lysates prepared from these cells were mixed with protein A-Sepharose beads coupled with anti-soMHVR (anti-HA MAb) and incubated at RT for 1 h. After washing, precipitated samples were electrophoresed in an SDS-polyacrylamide gel. The coimmunoprecipitated JHMV srr7 S proteins were then detected with an anti-MHV S protein MAb by ECL after Western blotting. The positions of the uncleaved S and S1 proteins in the lysate of DBT cells infected with JHMV srr7 (lane 9) are indicated. Arrowheads on the left indicate positions of molecular weight markers.

particular importance for understanding of the early events of viral infection and for the development of strategies to control or prevent infection. The soluble form of a viral receptor seems to be an ideal tool with which to analyze the initial phase of infection, especially the dynamics of viral entry into cells. Virus-receptor interactions have been studied in a number of systems using the soluble form of receptor (1, 3, 8, 10, 17, 24, 27, 28, 36, 40, 42).

Studies on the interaction of MHVR with the MHV S protein have identified the MHVR N-terminal region as a virus-binding domain (12, 15). They have also shown that the N-terminal region of the S protein (amino acids 1 to 330) interacts with MHVR (31, 45). However, the molecular events that occur during the early phase of infection remain largely unknown. Questions include the consequences of the receptor-MHV S protein interaction; whether the S protein undergoes the conformational changes after binding to the receptor; and the relationship of these changes to the activation of fusion activity.

In the present study, we have demonstrated soMHVR-mediated induction and enhancement of MHVR-independent fusion and infection. This effect was triggered by the interaction of S protein expressed on MHV-infected DBT cells with soMHVR. A consequence of this interaction was the activation of S protein-mediated fusion. In addition, we showed that soMHVR-mediated activation of the MHV S protein was possible in another system, i.e., S protein expressed on BHK cells

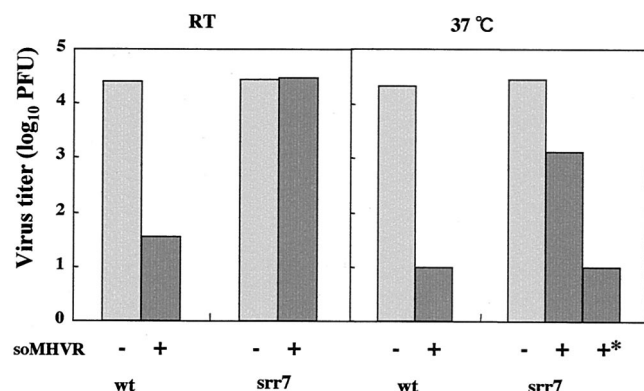


FIG. 7. Resistance of JHMV *srr7* to neutralization by soMHVR. wt JHMV or JHMV *srr7* (10^5 PFU in 25 μ l) was first mixed with the same volume of soMHVR containing 5,000 VN units (soMHVR +) or PBS (soMHVR -) and then incubated at RT or 37°C for 50 min, and residual virus infectivity was examined. Results for JHMV *srr7* mixed with soMHVR and incubated at 37°C for 2 h (soMHVR +*) are also shown. These data are representative of multiple independent experiments.

was also activated by soMHVR to induce or enhance syncytium formation. We believe that these observations mimic the early process of coronavirus infection. Importantly, this is the first demonstration that the fusogenic activity of the coronavirus S protein is activated by binding to the receptor protein. The activation of the MHV S protein by soMHVR, as demonstrated in this study, is very similar to the recent observations by Damico and Bates (8), who described the activation of avian sarcoma and leukosis virus (ASLV) envelope protein by its soluble receptor.

The soMHVR-mediated induction or enhancement of MHVR-independent fusion and infection suggests that virions treated with soMHVR should also be activated to infect BHK cells. We have tried in vain to infect BHK cells with MHV pretreated with soMHVR. This is in contrast to ASLV particles treated with soluble Tva receptor, which were activated to infect receptor-deficient cells (8). An important condition for this form of activation may be that the viral envelope protein bound by the soluble receptor is in close proximity to the target cell membrane. To obtain this situation in the ASLV system, a polycationic polymer and centrifugal forces were applied (8). In our MHVR-independent infection system, DBT cells expressing the MHV S protein on their surfaces were overlaid onto BHK cells and would be attached to the BHK cell surfaces. However, virions treated with soMHVR would not necessarily be in close contact with the target BHK cell surfaces. To place virions in close proximity to the BHK cell surface, we have cross-linked viruses with cells by use of concanavalin A. This protocol slightly enhanced soMHVR-mediated infection of BHK cells by MHV (unpublished data). The experiments reported here and our unpublished findings emphasize the two important roles played by the membrane-anchored receptor. First, it places the virus in close proximity to the cell membrane; second, it activates the viral protein so that the protein is able to induce the fusion of viral and cell membranes. Studies are currently in progress to efficiently position virions in

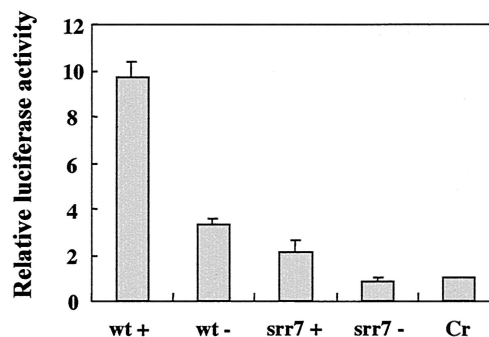


FIG. 8. Fusion enhancement by soMHVR as examined by luciferase activity. The first group of BHK cells was infected with vTF7.3 and then transfected with pTarget vectors containing either the wt JHMV S gene, the JHMV *srr7* S gene, or no S gene (Cr); then 5×10^5 cells were incubated in 24-well plates at 37°C for 7 h in the presence (+) or absence (-) of 150 nM soMHVR. The second group of BHK cells was transfected with pTM3-luc encoding the firefly luciferase gene, and 5×10^5 cells were overlaid onto the first group of BHK cells. After a 4-h incubation, luciferase activity in the culture was examined. Each data point (relative luciferase activity) represents the count of the sample divided by the count in cells transfected with a vector lacking the S gene. Error bars, standard deviations of three independent samples.

close proximity to MHVR-deficient cells in the presence of soMHVR.

In general, the soluble receptor neutralizes virus infectivity (3, 10, 17, 24, 27, 28). The neutralization may be due to the ability of the soluble receptor to compete with the membrane-anchored receptor for virus binding. Alternatively, the neutralization could be due to receptor-induced conformational changes of the envelope protein, which can no longer bind to the membrane-anchored receptor. Neutralization of HIV by soluble CD4 and of ASLV by soluble Tva is reportedly mediated by these mechanisms (3, 5). Neutralization of MHV with soMHVR also, most probably, results from the blockade of the binding of the virus to membrane-anchored MHVR (40, 42, 56), although the possibility that the S protein fails to bind to MHVR due to conformational changes has not been fully excluded. Since neutralization by the soluble receptor is highly efficient and mutants resistant to soluble receptor-mediated neutralization are difficult to obtain (compared, for example, to MAb escape mutants), the soluble receptor is a good candidate for therapeutic applications.

In contrast to the neutralization properties of the soluble receptor, soluble receptor-mediated activation of virus infectivity has been reported for HIV and simian immunodeficiency virus (SIV) (1, 7). A possible mechanism for this activation is that the envelope protein responsible for receptor binding and entry into cells is activated by the soluble receptor to interact with a coreceptor, and this occurs only after conformational changes of the protein mediated by binding to the receptor (43). However, soluble receptor-mediated viral activation has also been reported for ASLV, which does not require a second factor or coreceptor for the virus entry process (8). The activation of MHV S protein fusogenicity reported in this study is similar to that observed for ASLV rather than to that for HIV or SIV. The S protein activated by soMHVR acquires the ability to interact with receptor-deficient cells without requir-

ing a specific molecule, such as a coreceptor. A similar situation is evidenced by the binding of activated envelope protein with liposome (9, 23, 25).

We have reported that various MHV strains and mutants display MHVR-independent infection on BHK cells, although there was variation in the degree of infection (49). The most prominent infection was caused by JHMV strain cl-2. In contrast, JHMV srr7 had the least capability to infect by this mode. How JHMV cl-2 induces fusion in the absence of MHVR has not yet been clarified. Recently, Krueger et al. reported that dissociation of the JHMV S1 and S2 S protein subunits took place spontaneously after the S protein was synthesized in cells (30). It is possible that dissociation of S1 and S2 may be required to convert the S protein to the fusion-active form, and the propensity for different MHV S protein subunits to dissociate could be related to the ability to mediate receptor-independent infection.

The present study demonstrates that soMHVR activates the MHV S protein to execute fusion of MHV receptor-deficient BHK cells. Our current goal is to define the conformational change of the S protein, which presumably occurs following binding to soMHVR, and to relate these changes to the conversion from a nonfusogenic to a fusogenic form.

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